Development of Monoclonal Antibody-Based Competitive Immunoassays for the Detection of Picoxystrobin in Cereal and Oilseed Flours

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Abstract

Picoxystrobin is a new generation fungicide primarily developed to be used in cereal crops. In the present study a novel collection of specific monoclonal antibodies has been produced using different immunizing haptens based on a carboxy-functionalized identical spacer arm attached to alternative positions of the target pesticide molecule. Two competitive enzyme-linked immunosorbent assays have been developed employing hapten heterology, one using the antibody-coated direct format and the other in the conjugate-coated indirect format. Both immunoassays have been characterized in terms of selectivity, solvent tolerance, and buffer conditions, affording similar limits of detection at or below 0.1 µg/L. Finally, the optimized assays were applied to the analysis of picoxystrobin in wheat, corn, oat, barley, and soybean flours. Average recovery values from spiked samples were between 84 to 115%.

Keywords

ELISA, hapten, spacer arm, strobilurins, pesticide residues, food safety

1. Introduction

The intensive and occasionally abusive use of agrochemicals for crop yield improvement often leads to the presence of residual amounts of pesticides in cereals, possibly affecting the quality and safety of the final food product (Ruske, Gooding, & Dobrasczyk, 2004; Dornez et al., 2008). Picoxystrobin (PC) is a new broad-spectrum fungicide that belongs to the strobilurin family of pesticides and whose biological activity derives from the same *β*-methoxyacrylate toxophore group found in the natural active principle (strobilurin A) produced by the fungus S*trobilurus tenacellus* (Clough, 1993). The mechanism of action of PC is the inhibition of mitochondrial respiration by binding to the Q_0 site of cytochrome b, thus blocking electron transport between cytochrome b and cytochrome c_1 which eventually leads to disruption of the energy cycle (Bartlett et al., 2002). PC is effective against highly destructive pests in cereal crops such as *Septoria tritici*, *Leptosphaeria nodorum*, yellow rust, brown rust, ear diseases, and eyespot in wheat crops; net blotch, brown rust, powdery mildew, and *Rhychosporium* in barley crops; crown rust and powdery mildew in oat crops; and sclerotinia in oilseed rape (DuPont Global Website, 2011). This strobilurin fungicide shows preventive and curative properties, and it is currently formulated and commercialized under different trademarks by DuPont and Syngenta for cereal and oilseed crop protection (Bartlett, 2001; Balba, 2007).

According to EC regulation 396/2005, the maximum residue limit (MRL) for PC in most cereals and oilseeds is 50 µg/kg, whereas a specific MRL of 200 µg/kg has been established for barley and oat (European Commission, 2005). At present, diverse analytical methodologies are available for the analysis of PC residues in foodstuffs, most of which are based on liquid or gas chromatography coupled to mass spectrometry detectors. Pesticide extraction is usually carried out with different organic solvents such as acetone (Hiemstra & de Kok, 2007), ethyl acetate (Schurek et al., 2008; Taylor, Keenan,

Reid, & Uría-Fernández, 2008), or acetonitrile (Walorczyk & Gnusowski, 2009). In some cases, extraction was achieved by ultrasonic treatment combined with different clean-up approaches (Bo, Wang, Guo, Qin, & Lu, 2008; Campillo, Viñas, Aguinaga, Férez, & Hernández-Córdoba, 2010; Viñas, Martínez-Castillo, Campillo, & Hernández-Córdoba, 2010), whereas the application of solid-phase microextraction resulted in a substantial improvement in sensitivity for the determination of PC in baby foods (Viñas, Campillo, Martínez-Castillo, & Hernández-Cordoba, 2009).

As complementary analytical tools to chromatographic methods, a large number of studies based on enzyme-linked immunosorbent assay (ELISA) technology have been published for the determination of a wide variety of agrochemical residues in diverse food commodities (Morozova, Levashova, & Eremin, 2005; Van Emon, Chuang, Dill, & Xiong, 2008). Immunochemical methods are simple, rapid, and sensitive. In addition, intricate sample treatments are often not required, so direct dilution in an aqueous solution usually suffices for the analysis of liquid samples or for extracts of solid samples. Thus, ELISAs constitute not only a very useful routine laboratory approach where a large number of repetitive analyses need to be performed, but also a means for screening for the presence of specific compounds in a considerable assortment of samples. Moreover, since immunoassays can be implemented in many different formats, they can be adapted to a great variety of analytical circumstances. Nevertheless, the technique requires high-quality immunoreagents together with extensive and thorough characterization and validation studies.

Whilst ELISA is a well-established methodology for the detection and analysis of certain chemical contaminants in cereals such as mycotoxins (Skerritt, 1998; Goryacheva, Rusanova, Burmistrova, & De Saeger, 2009), few studies have been published for the determination of fungicide residues by enzyme immunoassay in those complex food

matrices (Danks, Chaudhry, Parker, Barker, & Banks, 2001; Jiang, Shi, Wu, & Wang, 2011). In previous publications, we described the syntheses of three PC derivatives functionalized with the same linker at rationally-selected sites of the target molecule (Parra et al., 2011) and the application of selected immunoassays to the analysis of PC residues in beer (Esteve-Turrillas et al., 2010). In the present study, novel monoclonal antibodies (mAb) to PC have been raised using regioisomeric PC haptens as immunogens. The affinity-purified antibodies were characterized in two competitive ELISA (cELISA) formats, the antibody-coated direct (d-cELISA) and the conjugate-coated indirect (i-cELISA) assays. In order to improve the limit of detection (LOD) of the selected immunoassays, the three available PC haptens were also evaluated as antigens in heterologous assays; i.e., assays employing conjugates carrying a different hapten to that employed for the generation of the antibody. A direct and an indirect cELISA were characterized and optimized for solvent tolerance and buffer conditions. Finally, both immunoassays were validated for the determination of PC residues present in different cereal and oilseed flours at concentration levels in accordance with the European MRLs for those foodstuffs.

2. Materials and methods

2.1 Reagents and instrumentation

Analytical-grade PC (methyl (E)-3-methoxy-2-{2-[6-(trifluoromethyl)-2-pyridyloxy methyl]phenyl}acrylate, CAS Registry No. 117428-22-5, MW 367.32 g/mol) and other employed pesticides were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). All pesticide standards were prepared as concentrated solutions in *N*,*N*-dimethylformamide (DMF) and were kept at −20 ºC in amber glass vials. Sephadex G-25 HiTrap Desalting columns and HiTrap Protein G HP columns from GE Healthcare (Uppsala, Sweden) were used for conjugate and antibody purification,

respectively. Polyclonal rabbit anti-mouse immunoglobulins conjugated to peroxidase (RAM–HRP) was from Dako (Glostrup, Denmark). Bovine serum albumin (BSA) fraction V and Hybridoma Fusion and Cloning Supplement were purchased from Roche Applied Science (Mannheim, Germany). HT (hypoxanthine–thymidine) and HAT (hypoxanthine–aminopterin–thymidine) supplements and gentamicine solution were obtained from Gibco BRL (Paisley, Scotland). Horseradish peroxidase (HRP), ovalbumin (OVA), *o*-phenylenediamine, cell culture media, fetal bovine serum, polyethylene glycol, amino acid solutions, Red Blood Cell Lysing Buffer Hybri-Max, and Freund's adjuvants were from Sigma-Aldrich (Madrid, Spain). P3-X63-Ag-8.653 mouse plasmacytoma cell line was from the European Collection of Cell Cultures (Wiltshire, UK). Culture plastic ware and Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY). ELISA plates were washed with an ELx405 microplate washer from BioTek Instruments (Winooski, VT) and the absorbance values were read in dual wavelength mode (492–650 nm) with a PowerWave HT device, also from BioTek Instruments.

Composition, concentration, and pH of the employed buffers were: (i) PB, 100 mM sodium phosphate buffer, pH 7.4; (ii) PBS, 10 mM sodium phosphate buffer, pH 7.4, with 140 mM NaCl; (iii) PBST, PBS containing 0.05% (v/v) Tween 20; (iv) 2×PBST, 20 mM sodium phosphate, pH 7.4, with 280 mM NaCl and 0.05% (v/v) Tween 20; (v) CB, 50 mM sodium carbonate–bicarbonate buffer, pH 9.6; (vi) Washing solution, 150 mM NaCl and 0.05% (v/v) Tween 20; (vii) Enzyme substrate buffer, 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4; and (viii) CitBT, 100 mM citrate buffer, pH 6.0, with 32 mM NaCl and 0.05% (v/v) Tween 20.

2.2 *Protein–hapten conjugates*

Three PC haptens (PCa6, PCb6, and PCo6) with the same linker at three alternative tethering sites were employed in the present study (Fig. 1). Those regioisomeric haptens contained a functional carboxylate group which was activated for coupling to the free amine groups of the carrier proteins. The synthesis of the PC derivatives and the preparation of the different conjugates were previously described (Parra et al., 2011; Esteve-Turrillas et al, 2010). BSA was used for immunogen preparation, OVA for conjugate-coated indirect assays, and HRP as enzyme tracer in d-cELISAs.

2.3 *Monoclonal antibody production*

Animal manipulation was performed according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Animals were immunized with BSA–PCb6 and BSA–PCo6 conjugates following equivalent procedures to those used in a previous study where the immunogen was BSA–PCa6 (Esteve-Turrillas et al., 2010). Briefly, sets of four mice each received three 200 µL intraperitoneal injections at three week intervals. Injections consisted of a 1:1 emulsion of a 1 mg/mL conjugate solution in PBS and Freund's adjuvant (complete for the first dose and incomplete for subsequent ones). After a resting period of at least 3 weeks from the last injection with adjuvant and four days before cell fusion, a booster injection in PBS was administered. For hybridoma production, murine myeloma cells and spleenocytes from immunized mice were fused using polyethylene glycol 1500. Next, hybrid cells were discriminated by employing aminopterin-containing supplement. Then, they were cloned by limiting dilution and cultured following published protocols (Mercader & Abad-Fuentes, 2009).

After cell fusion, a sequential double-screening strategy was applied in order to identify hybridomas producing high-quality antibodies. First, culture supernatants from the cell fusion experiment were screened by differential i-cELISA using plates coated with a 1000 ng/mL homologous OVA–hapten solution, as previously described (Abad et al., 1997). Within each set of mice immunized with a common conjugate, a low selection pressure (1000 nM PC as competitor) was applied in the screening of the first cell fusion, whereas subsequent hybridizations were assayed at this stage with 100 nM PC. Second, an additional screening was performed with the supernatants from those wells that afforded saturated signals in the first step. Checkerboard competitive indirect assays were carried out using microplates coated with 100 and 1000 ng/mL solutions of the homologous conjugate, serial dilutions of the supernatant, and higher selection pressure (lower PC concentrations, typically 5 and 50 nM) (Mercader, Suárez-Pantaleón; Agulló, Abad-Somovilla, & Abad-Fuentes, 2008). Following subcloning of the selected cell lines, immunoglobulins were purified from hybridoma culture supernatants by protein G affinity chromatography, and the antibodies were stored at 4 °C as ammonium sulfate precipitates. For daily usage, an aliquot was diluted in PBS containing 0.5% (w/v) BSA and 0.005% (w/v) thimerosal, and was kept cold in amber vials. The immunoglobulin isotype was determined using the Mouse MonoAb-ID kit (HRP labeled) from Invitrogen (Carlsbad, CA).

2.4 Direct cELISA procedure

Ninety-six-well polystyrene ELISA plates were coated with 100 µL per well of a 1000 ng/mL mAb solution in CB by overnight incubation at room temperature. Coated plates were washed four times with washing solution. PC standard curves were prepared in PBS as six-fold dilution series from a 0.9 g/L stock solution in DMF. The competitive immunological reaction was performed with 50 µL per well of standard or sample plus 50

µL per well of HRP tracer in PBST. After 1 h at room temperature, plates were washed again as described. Then, signal was produced by adding 100 µL per well of freshly prepared 2 mg/mL *o*-phenylenediamine and 0.012% (v/v) H_2O_2 in enzyme substrate buffer. Finally, the enzymatic reaction was stopped after 10 min at room temperature with 100 µL per well of 2.5 M sulfuric acid. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm.

2.5 Indirect cELISA procedure

Microplates were coated with 100 µL per well of a 100 or 1000 ng/mL OVA conjugate solution in CB by overnight incubation at room temperature. PC standards were prepared as described for the previous format. Coated plates were washed four times with washing solution, and they received 50 µL per well of standard or sample in PBS plus 50 µL per well of mAb in PBST. After 1 h incubation at room temperature, plates were washed again. Next, 100 µL per well of a 1/2000 dilution of RAM–HRP conjugate in PBST was added, and plates were incubated an additional 1 h at room temperature. Finally, after washing, signal was generated as described above for the direct cELISA procedure.

2.6 Buffer studies

Influence of Tween 20 concentration and buffer pH and ionic strength over the curve parameters of the selected assays was evaluated following a multiparametric approach (Esteve-Turrillas et al., 2010). Twenty buffers of different ionic strength, pH, and detergent concentration were assayed. Briefly, a central composite design was carried out consisting of a two-level full factorial design ($\alpha = 1.414$), with 3 factors and 3 replicates, which involved 8 cube, 6 axial, and 6 center points. Buffer conditions were fixed using NaCl, Tween 20, and three buffering systems: citrate ($pKa_2 = 4.8$, $pKa_3 = 6.4$), phosphate ($pKa_2 = 7.2$), and Tris (pKa = 8.1). PC standard curves were prepared in water and they

were mixed with the tracer (for direct competitive assays) or the mAb (for indirect competitive assays) diluted in every studied buffer. The responses using distinct conditions were fitted by a multiple regression equation, including curvature and interaction terms, using Minitab 14.1 software (Minitab Inc., State College, PA).

2.7 Data treatment

Raw or normalized absorbance values were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL). Assay sensitivity was estimated as the concentration value at the inflection point of the sigmoidal curve, typically corresponding to the analyte concentration affording a 50% inhibition (IC_{50}) of the maximum absorbance (A_{max}). The LOD was estimated as the concentration of PC that provided a 10% reduction of A_{max}, and cross-reactivity (CR) was calculated as the percentage of the ratio between the IC_{50} value for PC and the IC_{50} value for the corresponding pesticide (Fernández, Pinacho, Sánchez-Baeza, & Marco, 2011; Davies, 2005).

2.8 Sample processing and analysis

Wheat, corn, oat, barley, and soybean flours were purchased from local supermarkets. Five grams of flour were weighed in 15-mL centrifuge tubes and extracted with 10 mL of methanol using an ultrasonic bath for 10 minutes at room temperature. Then, samples were centrifuged at 2200×g for 5 min, and 40 µL of methanolic extract was diluted to 1 mL with Milli-Q water. For direct assays the tracer was diluted in CitBT, and for indirect assays the mAb was diluted in 2×PBST.

3. Results and discussion

3.1 Antigen and monoclonal antibody selection

Following standardized procedures for the generation of hybridoma cell lines, a collection of mAbs was eventually produced: four antibodies (PCa6#12, PCa6#13, PCa6#15, and PCa6#21) using the immunogen of PCa6 (Esteve-Turrillas et al., 2010), three (PCb6#11, PCb6#21, and PCb6#22) from hapten PCb6, and seven (PCo6#11, PCo6#13, PCo6#14, PCo6#16, PCo6#17, PCo6#18, and PCo6#21) from hapten PCo6. All of the available antibodies were of the IgG_1 isotype with κ light chains, except PCo6#14, which was an IgG_{2a}(λ). In order to select the best assay in each ELISA format, checkerboard competitive assays were performed by simultaneous evaluation of the fourteen purified mAbs in combination with the protein conjugates of the three synthetic haptens. The immobilized reagent (antibody or OVA conjugate for the direct or indirect format, respectively) was prepared at 100 and 1000 ng/mL, whereas the assayed concentrations of the reagent in solution (enzyme tracer or antibody for the direct or indirect format, respectively) during the competitive reaction were 10, 30, 100, 300, and 1000 ng/mL. Thus, a set of 10 inhibition curves was obtained for every pair of immunoreagents. For each antibody–antigen combination, Table 1 lists the IC_{50} and A_{max} values from the best inhibition curve in each ELISA format. The lower asymptotes were equivalent to the background in all cases. Remarkably, mAbs showing IC_{50} values to PC lower than 3 µg/L were derived from every immunizing hapten, regardless of the linker tethering site. Therefore, no hapten was shown to be inherently superior to others with respect to its suitability for the generation of high-affinity mAbs to PC. It was noticed that in the direct format most antibodies just recognized the homologous conjugate, whereas a more permissive binding to antigen structure variations was observed in the indirect format. For example, mAbs PCa6#12 and PCb6#21 bound all three OVA conjugates but

recognition was lost when heterologous tracers were used in the antibody-coated format. Concerning assay antigens, conjugates of PCa6 were bound just by homologous-derived antibodies, independently of the assay format, with the particular exception of mAb PCb6#21 in the i-cELISA format. In contrast, OVA–PCo6 was generally recognized by most of the antibodies, particularly in the i-cELISA format. The same result had been previously observed with an equivalent OVA conjugate of the strobilurins azoxystrobin and pyraclostrobin carrying the linker arm also at the methoxy group of the toxophore moiety (Parra, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, submitted; Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011). Probably, the many degrees of freedom of the toxophore moiety could explain the universal character as coating antigen of derivatives at this tethering site.

The CR of the mAbs towards other strobilurins and relevant pesticides was also assessed. The assayed compounds were: kresoxim-methyl, trifloxystrobin, pyraclostrobin, dimoxystrobin, azoxystrobin, fluoxastrobin, metominostrobin, orysastrobin, procymidone, boscalid, and fenhexamid. Calibration curves were prepared up to 2 µM in PBS; higher concentrations were not studied because of the poor water solubility of most pesticides. Low CR values was generally found (below 0.1%), and in those cases that some inhibition was observed with non-target compounds, the CR value was just slightly over 1%.

Several immunoassays with sensitivities in the low part-per-billion range were identified and re-evaluated (Table 1S in Supplementary Data). For further optimization and sample analysis, two assays were selected in different cELISA formats. First, a direct homologous assay with mAb PCb6#21 was chosen because of its elevated signal and low tracer consumption. Second, the assay with antibody PCa6#15 in combination with OVA–PCb6 was also selected because it afforded a highly specific detection of PC in the i-cELISA format.

3.2 *Assay characterization*

3.2.1 Solvent tolerance

Organic solvents such as acetone, acetonitrile, and methanol are usually employed for pesticide extraction from food samples. As a consequence, small amounts of solvents may be present during the immunological reaction which may affect the antibody–analyte interaction, thus providing inaccurate results. PC standard curves were prepared in PBS containing increasing amounts of acetone, acetonitrile, methanol, or ethanol. Concentrations of the assayed solvents equal or lower than 5% resulted in variations of the A_{max} values lower than 10% (results not shown). Concerning sensitivity, most solvents clearly increased IC_{50} values of both assays. The presence of up to 5% methanol in the standard curve resulted in an IC_{50} variation lower than 20%, making it the best tolerated solvent (Fig. 2).

3.2.2 Buffer conditions

A multiparametric study was performed in order to evaluate the robustness and stability of the selected assays upon changes in buffer properties, *i.e.*, ionic strength (*I*), pH, and surfactant concentration. Assayed *I* values ranged from 50 to 300 mM, pH was tested from 5.5 to 9.5, and the Tween 20 concentration was assayed from 0.00 to 0.05% (v/v). Central point conditions were pH 7.5, *I* = 163 mM, and 0.025% Tween 20, which are those of the assays described in the Materials and Methods section. The A_{max} and IC_{50} values of each PC inhibition curve that was obtained using every buffer were fitted by a multiple regression equation, including curvature and interaction terms. It was found that the influence of the detergent concentration over the curve parameters was not relevant (results not shown). Thus, a Tween 20 concentration of 0.025% in the assay was selected in order to prevent unspecific interactions with the sample matrix.

Variations of pH and ionic strength resulted in dissimilar effects over the inhibition curve parameters of the two studied ELISAs. Fig. 3 shows the surface plots depicting changes of A_{max} and IC_{50} values as a function of buffer ionic strength and pH. In the case of the d-cELISA, based on mAb PCb6#21, the A_{max} increased at higher ionic strength and at low pH, whereas the IC_{50} had a maximum at the central point and it decreased at extreme values. Consequently, phosphate buffer conditions were deemed suboptimal for this ELISA, as results indicated that sensitivity could be enhanced using a buffer of higher ionic strength and lower pH. Hence, a 300 mM citrate buffer, pH 6.0, with 0.025% Tween 20 (v/v) was proposed for further studies. As expected, when PC standard curves were run in parallel under preliminary (phosphate) and optimized (citrate) conditions, a 10% reduction of the IC_{50} value was achieved. Regarding the i-cELISA with mAb PCa6#15, the A_{max} showed a maximum value close to the central point, and the IC_{50} was minimal with ionic strengths from 180 to 240 mM at any pH. Therefore, this model predicted that phosphate was an adequate buffer to obtain the best performance in this immunoassay.

Fig. 4 shows the inhibition curves obtained for both optimized ELISAs. The d-cELISA was carried out with microplates coated using a 1000 ng/mL PCb6#21 solution and a 50 ng/mL HRP–PCb6 solution in CitBT. The i-cELISA employed plates that had been coated with a 1000 ng/mL OVA–PCb6 solution and 80 ng/mL of mAb PCa6#15 in 2×PBST. PC standards were prepared in Milli-Q water by serial dilution in both cases. The theoretical LODs of the developed ELISAs were 0.10 µg/L and 0.07 µg/L for direct and indirect assays, respectively. Low variability was found for both ELISAs; intra-assay coefficients of variation (three replicates) of the analytical standards in the dynamic range (20-80% signal) of the inhibition curves were below 4%, whereas inter-assay coefficients of variation (three replicates) were below 6%.

328 ### *3.3 Determination of picoxystrobin in flour samples*

329 #### *3.3.1 Matrix effects*

330 Wheat, corn, oat, barley, and soybean flours were selected as relevant commodities
331 to evaluate the applicability of the proposed ELISAs. Methanol was chosen for extraction
332 because it was reasonably well-tolerated by the developed cELISAs (see above), and its
333 efficiency for the extraction of pesticides from wheat flour had been previously
334 demonstrated (Skerritt, Guihot, Hill, Desmarchelier, & Gore, 1996). Analytical procedures
335 for the determination of pesticide residues in food by cELISA often require a dilution step
336 in order to avoid solvent interferences and matrix effects. Flour methanolic extracts were
337 diluted with Milli-Q water and employed to prepare PC standard curves. The matrix
338 influence was evaluated independently for each of the five flour samples, and the five
339 curves for each dilution were averaged. Low dilutions (1/10) had variable effects over the
340 curve parameters depending on the flour type so the average curve had a large dispersion
341 (Fig. 1S in Supplementary Data). However, a 1/100 dilution of the flour extracts
342 significantly minimized matrix effects, thus resulting in inhibition curves essentially
343 overlaying that obtained in buffer.

344 #### *3.3.2 Recovery studies*

345 Flour samples were spiked with fixed amounts of PC and extracted with methanol,
346 diluted as described with Milli-Q water, and measured by the two developed cELISAs
347 under the optimized conditions. Unspiked flour extracts were also run in every experiment
348 as negative controls. PC determinations in oat and barley flours were accurate with both
349 cELISAs at concentrations covering by far the MRLs (200 µg/kg) for these foodstuffs
350 (Table 2). Regarding wheat, corn, and soybean flours, higher interferences were observed
351 with the direct ELISA at the MRL (50 µg/kg), but the recovery yields achieved with the

indirect assay were acceptable at this concentration. In general, the indirect assay afforded better recovery values at low PC concentrations than the direct assay. Average recovery values ranged from 101 to 115% with the d-cELISA and from 84 to 112% with the i-cELISA.

In summary, novel mAbs to PC were produced using regioisomeric synthetic haptens carrying the same spacer arm tethered to alternative positions of the target molecule. When employed as immunogens, each of those haptens allowed the generation of mAbs with good affinity and selectivity to PC. This collection of mAbs showed a more restrictive binding profile to heterologous conjugates than the polyclonal antibodies obtained in a previous study using the same haptens (Parra, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011). In addition, two competitive immunoassays using either the antibody-coated d-cELISA or the conjugate-coated i-cELISA format were developed, with LODs at or below 0.1 µg/L. When the optimized assays were applied to methanolic extracts of wheat, corn, oat, barley, and soybean flours fortified with PC at concentrations in line with the European MRLs for this pesticide in those food commodities, satisfactory recoveries were obtained after a simple dilution in water, particularly with the indirect assay format.

Abbreviations

BSA, bovine serum albumin; cELISA, competitive enzyme-linked immunosorbent assay; CR, cross-reactivity; d-cELISA, direct cELISA; DMF, *N*,*N*-dimethylformamide; HRP, horseradish peroxidase; i-cELISA, indirect cELISA; LOD, limit of detection; mAb, monoclonal antibody; MRL, maximum residue limit; OVA, ovalbumin; PC, picoxystrobin; RAM–HRP, polyclonal rabbit anti-mouse immunoglobulins conjugated to peroxidase. For

buffer abbreviations and composition and for the definitions of A_{max} and IC_{50} see the Materials and methods section.

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Limited amounts of the described immunoreagents are available upon request.

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Figure captions

Fig. 1. Structure of picoxystrobin and of the three regioisomeric synthetic haptens employed for mAb production and assay development.

Fig. 2. Effect of solvent concentration on the sensitivity (IC_{50}) of the two selected ELISAs. Methanol (circles), ethanol (triangles down), acetonitrile (squares), and acetone (diamonds).

Fig. 3. Surface plots of the A_{max} and IC_{50} values as a function of buffer pH and ionic strength (*I*) for the d-cELISA and the i-cELISA.

Fig. 4. Picoxystrobin standard curves obtained with the optimized cELISAs. Values are the mean of three independent experiments. The homologous direct assay with mAb PCb6#21 (triangles up) afforded an assay whose slope and IC_{50} values were −1.06 ± 0.02 and 0.86 ± 0.03 µg/L, respectively, whereas the indirect assay using mAb PCa6#15 and OVA–PCb6 (circles) had a slope of −1.08 ± 0.03 and an IC_{50} value of 0.94 ± 0.03 µg/L. The A_{max} values were between 1.0 and 1.5.

512

Table 1
Figures of merit from the most sensitive immunoreagent combinations as evaluated by checkerboard direct and indirect competitive experiments.

	Direct assay						Indirect assay					
	HRP–PCa6		HRP–PCb6		HRP–PCo6		OVA–PCa6		OVA–PCb6		OVA–PCo6	
mAb	A_{max}	IC_{50} (µg/L)	A_{max}	IC_{50} (µg/L)	A_{max}	IC_{50} (µg/L)	A_{max}	IC_{50} (µg/L)	A_{max}	IC_{50} (µg/L)	A_{max}	IC_{50} (µg/L)
PCa6#12	2.31	5.15	-[a]	-	-	-	1.20	2.22	0.69	2.28	1.18	3.18
PCa6#13	1.61	2.24	-	-	1.23	3.42	1.78	1.38	-	-	1.55	1.77
PCa6#15	2.05	2.32	1.23	0.89	-	-	1.55	2.62	1.70	1.03	-	-
PCa6#21	1.14	7.86	1.44	0.83	-	-	1.18	0.89	1.94	1.05	1.10	0.94
PCb6#11	-	-	1.64	1.52	-	-	-	-	1.72	3.13	1.14	0.88
PCb6#21	-	-	1.90	0.94	-	-	1.80	3.54	2.11	1.17	1.41	2.62
PCb6#22	-	-	2.02	3.33	-	-	-	-	1.67	1.51	-	-
PCo6#11	-	-	-	-	1.04	2.76	-	-	-	-	0.98	1.41
PCo6#13	-	-	-	-	1.12	2.00	-	-	-	-	0.94	1.13
PCo6#14	-	-	-	-	1.13	3.20	-	-	-	-	1.36	4.25
PCo6#16	-	-	0.59	4.26	-	-	-	-	0.83	2.57	1.52	6.25
PCo6#17	-	-	-	-	-	-	-	-	-	-	2.39	4.80
PCo6#18	-	-	-	-	-	-	-	-	-	-	1.29	10.87
PCo6#21	-	-	1.50	1.08	1.23	2.54	-	-	1.16	1.75	1.59	2.01

[a] The highest A_{max} observed for these combinations was below 0.5.

Table 2

Recovery values obtained with the proposed immunoassays from flour extracts spiked with picoxystrobin.

	[PC] in the flour	recovery (% ± s, n=3)				
assay	(µg/kg)	wheat	corn	oat	barley	soybean
d-cELISA using	25	-[a]	-	-	-	-
mAb PCb6#21 and	50	127 ± 32	139 ± 10	136 ± 2	140 ± 5	139 ± 6
HRP–PCb6	100	130 ± 9	126 ± 6	121 ± 5	120 ± 1	105 ± 3
	250	109 ± 11	106 ± 4	98 ± 6	101 ± 5	93 ± 4
	500	104 ± 3	107 ± 7	100 ± 5	95 ± 3	91 ± 4
	1000	99 ± 6	98 ± 5	85 ± 6	85 ± 8	77 ± 7
	Average	114 ± 14	115 ± 17	108 ± 20	108 ± 22	101 ± 23
i-cELISA using	25	101 ± 9	99 ± 9	74 ± 25	120 ± 35	134 ± 9
mAb PCa6#15 and	50	127 ± 15	121 ± 11	88 ± 9	106 ± 26	122 ± 11
OVA–PCb6	100	110 ± 4	104 ± 13	93 ± 8	107 ± 13	113 ± 6
	250	112 ± 3	110 ± 8	86 ± 10	107 ± 6	92 ± 10
	500	118 ± 7	118 ± 8	80 ± 3	101 ± 7	89 ± 13
	1000	105 ± 5	98 ± 6	80 ± 25	96 ± 11	73 ± 9
	Average	112 ± 9	108 ± 10	84 ± 7	106 ± 8	104 ± 23

[a] Below assay detectability.

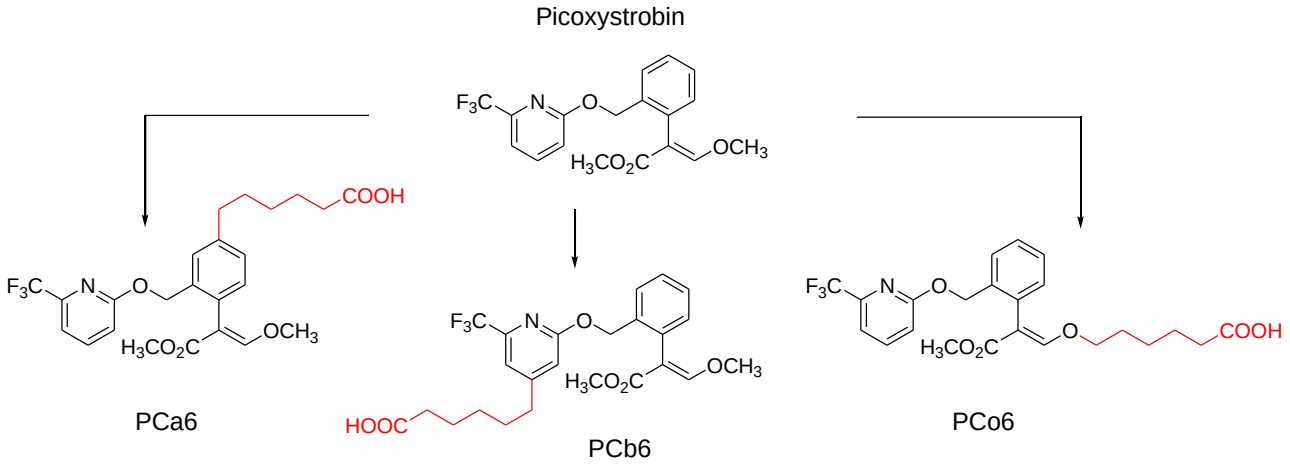

Fig. 1. Mercader et al.

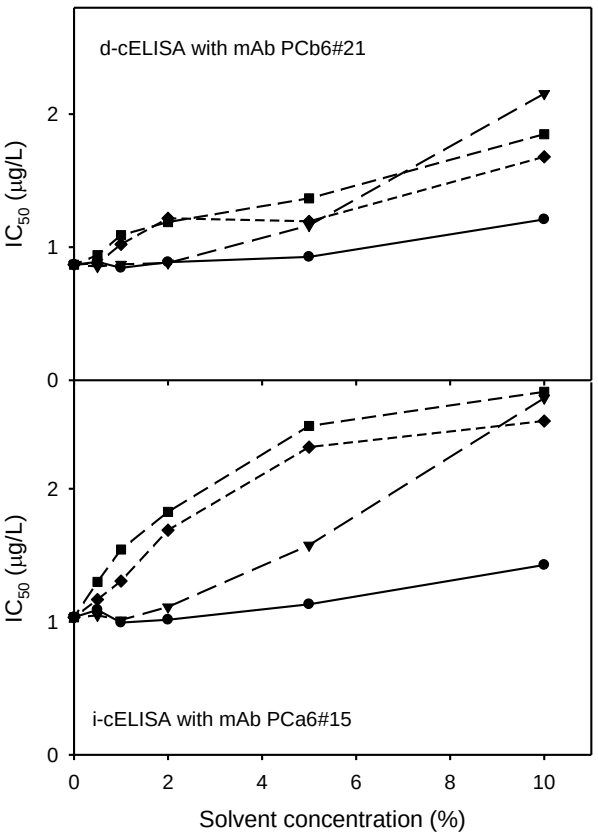

Fig. 2. Mercader et al.

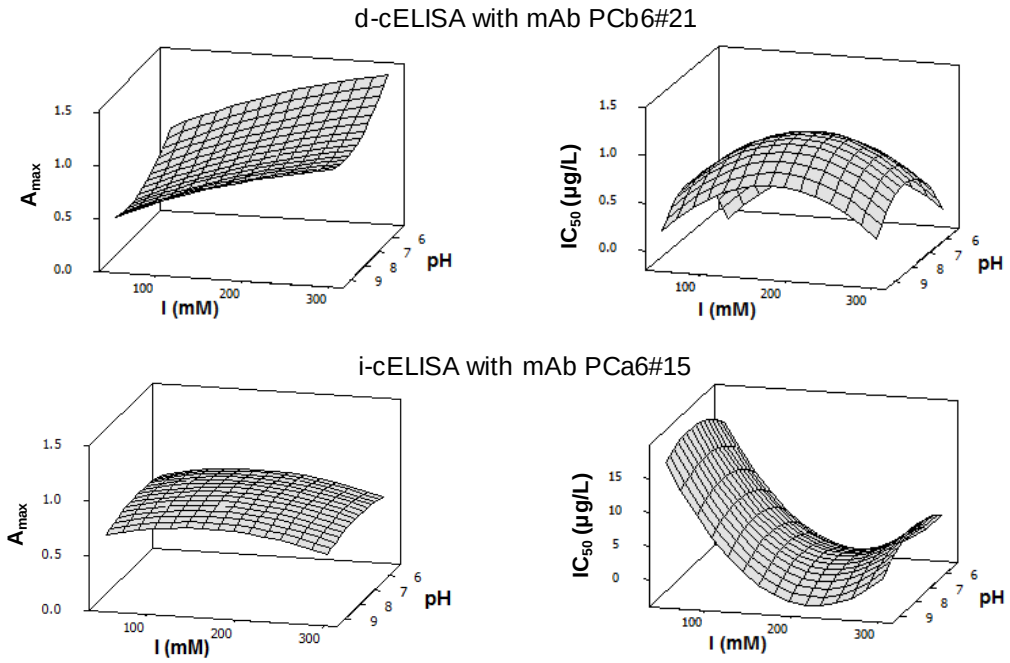

Fig. 3. Mercader et al.

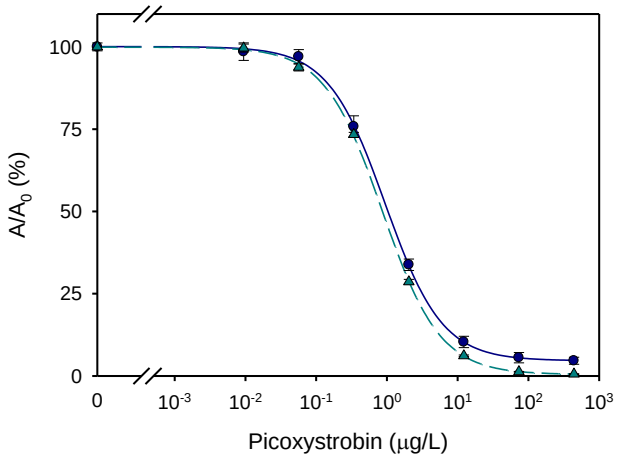

Fig. 4. Mercader et al.